RTA®

HIV-1 Real-Time PCR Kit

Handbook

Date of Issue –- 2011-04

IVD

Quantitative detection of Human Immunodeficiency Virus type 1 RNA

For *in vitro* diagnostic use

For use with the Stratagene MX3000p/MX3005p and

INCEPTRA Cycler 4840/9620/9640/9660/9680 Instruments

For professional use only

REF 09013025 – 25 tests
09013100 – 100 tests

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Kit Contents

	Cap Color	Reagents	25 tests	100 tests
1	BROWN	HIV-1 Reaction Mix	500 µl	2x1000 µl
2	YELLOW	HIV-1 Enzyme Mix	33 µl	130 µl
3	BLUE	HIV-1 Internal Control	250 µl	250 µl
4	RED	HIV-1 Quantification Standart 1 (10^7 IU/ml)	200 µl	200 µl
5	RED	HIV-1 Quantification Standart 2 (10^6 IU/ml)	200 µl	200 µl
6	RED	HIV-1 Quantification Standart 3 (10^5 IU/ml)	200 µl	200 µl
7	RED	HIV-1 Quantification Standart 4 (10^4 IU/ml)	200 µl	200 µl
8	WHITE	PCR Grade Water	1000 µl	1000 µl

Storage

All reagents of RTA HIV-1 Real-Time PCR Kit should be stored at -20°C. Storage at higher temperatures should be avoided (e.g. +4°C). Under these conditions, kit contents should be stable through the expiration date printed on the label. The reagents should not be freeze-thawed more than 2 times; otherwise the shelf of the kit will reduce. During the working steps all reagents should be kept on ice.

Intended Use

RTA HIV-1 Real-Time PCR Kit is an in vitro nucleic acid amplification assay for quantification of Human Immunodeficiency virus type 1 (HIV-1) RNA in human serum or plasma (EDTA) using RTA Viral Nucleic Acid Isolation Kit and Stratagene Mx3000p/Mx3005p instrument or INCEPTRA Cycler 4840/9620/9640/9660/9680 instrument. RTA HIV-1 Real-Time PCR Kit is intended for use as an aid in the management of patients with chronic HIV-1 infection undergoing anti-viral therapy to assess response to treatment in conjunction with all relevant clinical and laboratory findings. RTA HIV-1 Real-Time PCR Kit is not intended for screening of blood and blood products for the presence of HIV-1 RNA or confirmation of the diagnosis of infection with HIV-1.

Product Use Limitations

All reagents of the kit are for in vitro diagnostic use only.

RTA HIV-1Real-Time PCR Kit is not intended for screening of blood and blood products for the presence of HIV-1 RNA or confirmation of the diagnosis of infection with HIV-1.

This kit has been validated for use with human serum or human plasma collected in EDTA anticoagulant. Test with other sample types may result in inaccurate results.

This kit has been validated for use with RTA Viral Nucleic Acid Isolation Kit. Using other isolation kits may adversely affect the performance characteristics of the kit.

This kit has been validated for use with Stratagene Mx3000p/Mx3005p instrument or INCEPTRA Cycler 4840/9620/9640/9660/9680. Using other instruments may adversely affect the performance characteristics of the kit.

Trustworthy results depend on proper sample collection, transport, storage and processing methods.

It is intended for professional use by properly trained personnel.

RTA HIV-1 Real-Time PCR Kit is intended for use as an aid in the management of patients with chronic HIV-1 infection undergoing anti-viral therapy to assess response to treatment in conjunction with all relevant clinical and laboratory findings.

The instructions in user manual should be followed strictly for optimum PCR results.

The expired kits should not be used. Kit components from different lots should not be mixed.

Product Description

RTA HIV-1 real-time PCR assay is a one step real time reverse transcription PCR assay in which RNA templates are first reverse-transcribed to generate complementary cDNA strands followed by DNA polymerase-mediated cDNA amplification.. During cDNA replication in the PCR process, the internal oligonucleotide hybridizes to the template and is digested by the 5'-3' endonuclease activity of the Thermus aquaticus (Taq) DNA polymerase as the PCR primer is extended. The internal oligonucleotide is digested only if cDNA replication occurs, separating the fluorescent and quencher molecules. PCR products are detected within minutes by monitoring the increase in fluorescence that occurs exponentially with successive PCR amplification cycles. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus Ct is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring Ct and using the standard curve to determine starting copy number. RTA HIV-1 real-time PCR assay utilizes external standards to gather

quantitative results and includes an internal control, which controls for target isolation and amplification.

Pathogen Information

HIV is classified in the family Retroviridae, subfamily Lentivirinae, and genus Lentivirus. The structure of HIV follows the typical pattern of the retrovirus family, comprising a singlestranded, positive-sense ribonucleic acid (RNA) genome of about 9.7 kilobases. There are two strands of HIV RNA and each strand has a copy of the virus's nine genes. The RNA is surrounded by a cone-shaped capsid which consists of approximately 2000 copies of the p24 viral protein. Surrounding the capsid is the viral envelope. The viral envelope is composed of a lipid bilayer membrane, formed from the cellular membraneof the host cell during budding of the newly formed virus particle. Host-cell proteins, such as the major histocompatibility complex (MHC) antigens and actin, remain embedded within the viral envelope, along with the viral envelope protein. Each envelope subunit consists of two non-covalently linked membrane proteins: glycoprotein (gp) 120, the outer envelope protein, and gp41, the transmembrane protein that anchors the glycoprotein complex to the surface of the virion. The envelope protein is the most variable component of HIV, although gp120 itself is structurally divided into highly variable (V) and more constant (C) regions. The variability of V regions may be a product of envelope functionality, as has been especially well described in V3, where amino acid changes alter coreceptor use. The variability of the HIV envelope also confers a uniquely complex antigenic diversity.

HIV-1 is divided into three quite distinct lineages: the groups M, N and O. Again, the worldwide distribution of these groups is not equal: group M (for Main) strains are substantially more common in the global epidemic than the group O (Outlier) strains, which are largely confined to Africa, with sporadic cases reported elsewhere. The group N (non-M, non-O) strains have only been isolated in Cameroon.

Warnings and Precautions

All clinical specimens and all resulting waste materials should be treated as potentially infectious; the samples should be prepared in Bio-safety Level 2 area .

Before and after work all surfaces should be disinfected with a freshly prepared solution of 10% bleach or antiviral agents.

Dispose of unused reagents, waste and specimens in accordance with country or local regulations.

Do not pipette by mouth.

Do not eat, drink or smoke in laboratory work areas.

Wear protective disposable gloves, laboratory coats and eye-wear when handling clinical specimens and kit reagents. Wash hands thoroughly after handling specimens and test reagents.

Avoid contact of reagents with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water,

The procedures should preferably be performed in four separated areas (i.e. for RNA extraction, PCR setup, sample addition, amplification) to aid in preventing contamination.. All supplies for a particular procedure should be stored in the area where that procedure is performed and should not be moved between areas Gloves should be removed and disposed of before leaving one area to proceed to the next. Lab coats should be specific to an area and never worn outside of that area. The work should flow in one direction, beginning in the extraction area, moving to the PCR setup area in which PCR Master Mix is prepared , then moving to the third area in which samples, negative control and quantification standards are added, finally moving to amplification area in which real-time PCR equipment is run.

Warnings and Precautions *(continued)*

Use all pipetting devices and instruments with care and follow the manufacturer's instructions for calibration and quality control; to prevent sample contamination, use new, sterile aerosol barrier or positive displacement RNase-free pipette tips and sterile pipettes.

Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.

Store the kit away from any source of contaminating DNA or RNA, especially amplified nucleic acid.

Do not mix reagents with different lot numbers or substitute reagents from other manufacturers.

A single type of HIV-1 RNA assay should be used for monitoring a patient. If RTA HIV-1 Real-Time PCR Kit substitutes another HIV-1 RNA assay, both tests should be used in parallel for at least two subsequent samples.

Do not use a kit after its expiration date.

Additional Materials Required

- RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029; RTA Laboratories, Turkey)
- Real-Time PCR system
- Disposable powder-free gloves
- Micropipettes (0.5 µl – 1000 µl)
- Sterile micropipette tips with filters
- Microcentrifuge tubes
- Vortex mixer
- Desktop microcentrifuge for 2.0 ml tubes and for PCR strip tubes
- Real-Time PCR reaction tubes/plates/capillaries
- PCR workstation

Sample Preparation

This kit has been validated for use with human serum or human plasma collected in EDTA anticoagulant.

Aseptic techniques must be employed during collection to prevent the introduction of micro-organisms into the patient's anatomical space, and to prevent the sample from being contaminated during the process of collection.

All samples should be regarded as potentially infectious and standard precautions guidelines should be followed by all healthcare workers during sample collection and handling.

Samples must be collected into appropriate containers before despatch to the laboratory.

Be careful to check for cracks in the containers and to ensure that the lids of containers are properly tightened to prevent leakage of samples during handling and transportation. This can pose infection hazards to transport and laboratory staff.

Ensure that the outer surfaces of the containers are not contaminated by the patients' samples.

Store whole blood at room temperature for no longer than 4 hours. Centrifuge blood within 4 hours of collection.

Transfer serum or plasma to a screw cap cryovial tube.

Transportation of whole blood, serum or plasma must conform to country or local regulations for the transport of etiologic agents.

Serum or plasma samples may be stored at 2-8°C for up to 3 days or frozen at -70°C or colder for long-term storage.

Avoid multiple freeze/thaw cycles of specimens.

Protocol

Viral RNA Isolation

RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029; RTA Laboratories, Turkey) should be used for viral RNA extraction from clinical samples. Please follow the manufacturer's instructions as stated in the kit manual.

Internal Control

During RNA isolation, addition of the supplied internal control (IC) is necessary. IC allows the user to monitor RNA extraction step as well as to determine any PCR inhibition. For each sample, add 2.5 µl IC together with Solution RL of the Viral Nucleic Acid Isolation Kit for a 50 µl elution. Depending on your final elution volume, the volume of IC to be added can be calculated (0.05 µl IC/1 µl Elution Buffer). There was no amplification of internal control in the tests where high positive HIV-1 samples were amplified because there was a competition between internal control template and HIV-1 RNA template for using PCR primers and other components. The Ct value of internal control of a negative sample should be equal to 33 ± 5, otherwise,it denotes a problem during purification.

Quantification Standards

For generating a standard curve to obtain accurate quantification data on the Real-Time system, four quantification standards should be used. For each standard the corresponding concentration should be defined properly to the Real-Time PCR system before each run and the standard curve will be generated accordingly at the end of the reaction. Work with HIV-1 Quantification Standards after preparation of clinical samples and negative control in a separate area. Caps of the tubes or capillaries of Clinical Samples SHOULD be closed in that area

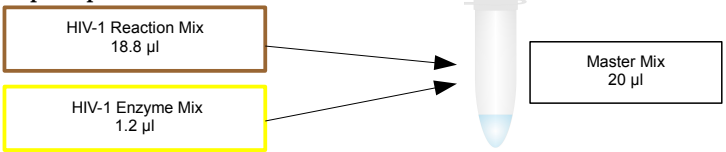
Protocol
(continued)

PCR Protocol

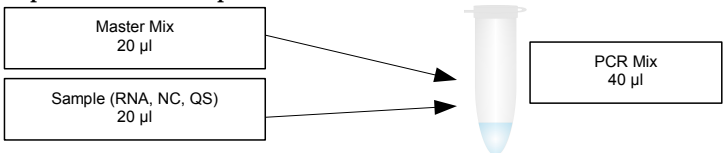
- Thaw all components, except HIV-1 Enzyme Mix, at room temperature. Thaw HIV-1 Reaction Mix at 37°C for 5 min if there is a precipitate. Put HIV-1 Enzyme Mix on ice. Mix each component thoroughly, then centrifuge briefly before use. Transfer all the reagents onto ice or cooling block.
- The final volume of Master Mix is calculated by multiplying single reaction volumes of Reaction Mix and Enzyme Mix by the total sample size. The number of negative controls, quantification standards and the clinical samples should be included when calculating total sample size. Against possible pipetting errors, addition of an extra sample to the total sample size is recommended. PCR Grade Water should be used as the negative control.
- To prepare master mix, add 18.8 µl of HIV-1 Reaction Mix (brown tube) and 1.2 µl of HIV-1 Enzyme Mix (yellow cap) for each sample to the master mix tube. Vortex the tube and spin down briefly in a microcentrifuge. Add 20 µl of Master Mix into Real-Time PCR reaction tubes or capillaries for each sample. Add 20 µl RNA of each sample, negative control and quantification standards into the tubes. Spin down briefly.
- Perform the following protocol for Stratagene MX3000p/MX3005p: 45°C for 30 min,95°C for 10 min, 1 cycle; 95°C for 30 sec, 63°C for 60 sec, 72°C for 30 sec, 45 cycles.
- Perform the following protocol for INCEPTRA Cycler 4840/9620/9640/9660/9680: 45°C for 30 min, 95°C for 10 min, 1 cycle; 95°C for 30 sec, 60°C for 60 sec, 72°C for 30 sec, 45 cycles
- Fluorescence is measured at 72°; FAM and HEX channels should be chosen (See the schema in the next page).
- Refer to the Operator's Manual of the related instruments to program and analyze the results.
- During analysis on STRATAGENE software, adjust threshold fluorescence value manually by entering 1000 for FAM and 500 for HEX.
- During analysis on INCEPTRA Cycler software, adjust baseline value manually by entering 100 for both FAM and HEX.

Protocol

1. Step: Preparation of Master Mix



2. Step: Addition of Samples



3. Step: Programming of Thermal Cycler

Program Name	Cycles	Program for Stratagene	Program for INCEPTRA
CDNA Synthesis	1	45ºC, 30 min	45ºC, 30 min
Hot Start	1	95ºC, 10 min	95ºC, 10 min
Amplification*	45	95ºC, 30 sec	95ºC, 30 sec
		63ºC, 60 sec	60ºC, 60 sec
		72ºC, 30 sec	72ºC, 30 sec

** Fluorescence is measured at 72°C;FAM and HEX/VIC/JOE channels should be chosen*

Data Analysis

Following results can be interpreted:

➢If a signal is detected in FAM channel , it is a **POSITIVE** result. Concentration of each positive sample will be calculated by the software according to the standard curve as International Unit per milliliter (IU/ml) . Due to different starting sample volumes and elution volumes during viral DNA isolation, the following formula **SHOULD** be used to calculate the concentration of the original clinical sample:

$$\textbf{Concentration of the Original Sample (IU/ml)} = \frac{\textbf{Concentration from Software (IU/ml)} \times \textbf{Elution Volume (µl)}}{\textbf{Original Sample Volume (µl)}}$$

➢If no signal is detected in FAM channel (Ct≥40) and a signal is detected in HEX channel (Ct= 33 ± 5) , it is a **NEGATIVE** result.

➢If no signal is detected in FAM channel (Ct≥40) and HEX channel (Ct>38), **NO DIAGNOSTIC INTERPRETATION** can be done.

Data Analysis

Following table shows typicval Ct values of Quantification Standards for different real time-PCR systems

	Expected Ct values			
INSTRUMENTS	**Quantification Standard 1 (10^7 IU/ml)**	**Quantification Standard 2 (10^6 IU/ml)**	**Quantification Standard 3 (10^5 IU/ml)**	**Quantification Standard 4 (10^4 IU/ml)**
STRATAGENE Mx3000p/Mx3005p	23.60±2	26.70±2	30.00±2	33.40±2
INCEPTRA Cycler	23.50±2	27.00±2	30.00±2	33.00±2

Performance Characteristics

Analytical Sensitivity

Analytical sensitivity was analyzed by use of a dilution series of WHO standard, and the cutoff value of the kit was determined by probit analysis for STRATAGENE Mx3000p and INCEPTRA Cycler 9660 Real-Time PCR Systems. A dilution series of a WHO International HIV-1 standard (NIBSC code: 97/650) was prepared to give the final concentrations of 1000, 500, 200, 100, 50 and 25 IU/ml. Dilutions were extracted by RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029) according to RTA Viral Nucleic Acid Isolation Kit Handbook. Starting sample volumes were 500 µl and elution volumes were 50 µl. Each dilution was tested in 24 replicates for each instrument. Lower limit was calculated by probit analysis done by PASW Statistics 18 program. The 95 % cutoff concentration of RTA HIV-1 Real-Time PCR Kit is 56 IU/ml for STRATAGENE and 85 IU/ml for INCEPTRA Cycler.

	INCEPTRA Cycler	STRATAGENE
RTA Viral Nucleic Acid Isolation Kit (Starting sample volume=500 ul, Elution volume=50 ul)	85 IU/ml	56 IU/ml

Performance Characteristics

Linear Range

To determine the upper limit, a dilution series of Acrometrix OptiQuant HIV-1 RNA Quantification Panel (Cat. no: 942013) ranging from 1 x 10^4 IU/ml to 1 x 10^6 IU/ml were prepared.Viral RNA was extracted from standards by RTA Viral Nucleic Acid Isolation Kit (Cat No: 09029) according to RTA Viral Nucleic Acid Isolation Kit Handbook. Starting sample volumes were 500 µl and elution volumes were 50 µl. High concentration samples (1 x 10^7, 1 x 10^8 and 1 x 10^9 IU/ml) were prepared by using calibrated in vitro transcribed RNA bearing HIV-1 quantification standard. Within this range, the relationship between log of target RNA and Ct values is linear. Linear regression analyses comparing the Ct values-versus- log of target RNA were as follows:

For STRATAGENE, Ct value = -3.304(log of target RNA) + 43.85; with a correlation coefficient (R^2) of 0.998.

For INCEPTRA Cycler, Ct value = -3.22(log of target RNA) + 44.00; with a correlation coefficient (R^2) of 0.994.

Upper limit is at least 1 x 10^9 IU/ml for STRATAGENE and INCEPTRA Cycler.

Lower limit was calculated by probit analysis done by PASW Statistics 18 program according to the quantification results of HIV-1 Analytical Sensitivity Studies. 95 % lower confidence limit is 58.7 IU/ml for STRATAGENE and 85 IU/ml for INCEPTRA Cycler. Dynamic ranges of RTA HCV Real-Time PCR Kit:

For STRATAGENE **58.7 - 1 x 10^9 IU/ml**

For INCEPTRA Cycler **85 - 1 x 10^9 IU/ml**

Performance Characteristics

Precision

For each experiment, 24 replicates of 10^5 IU/ml HIV-1 RNA, 2nd WHO International Standard (NIBSC code: 97/650) were used. Intra-assay precision associated with Ct values was 1.34 % of variation coefficient for 10^5 IU/ml of target RNA. Inter-assay precision associated with Ct values was 0.8 % of variation coefficient for 10^5 IU/ml of target RNA. Inter-batch precision associated with Ct values was 0.8 % of variation coefficient for 10^5 IU/ml of target RNA. Precision for INCEPTRA Cycler 9660 instrument associated with Ct values was 2,03 % of variation coefficient for 10^5 IU/ml of target RNA. Precision for LightCycler 2.0 instrument associated with Ct values was 0,20 % of variation coefficient for 10^5 IU/ml of target RNA And overall precision associated with Ct values was 3,15 % of variation coefficient for 10^5 IU/ml of target RNA The results on basis of Ct values are shown in the following table:

	N	Mean	Std. Deviation	Variance	Coefficient of variation (%)
Intra_assay	24	32,1046	,43229	,187	1,34
Inter_assay	24	31,6471	,25503	,065	0,8
Inter_batch	24	31,6621	,25228	,064	0,8
LightCycler	24	29,9063	,06378	,004	0,2
Inceptra	24	30,0813	,61747	,381	2,03
TOTAL	120	31,0803	,97949	,959	3,15

Performance Characteristics

Genotype Detectability

The performance of RTA HIV-1 Real-Time PCR Kit was evaluated with SeraCare Life Sciences HIV-1 RNA Genotype Performance Panel. As a result of this study, RTA HIV-1 Real-Time PCR Kit can detect and quantify all members of Major group of HIV-1 genotypes (A, B, C, D, AE, F, AG, G, H).

Diagnostic Specifity

HIV-1 negative clinical specimens were analyzed to determine the diagnostic specificity of RTA HIV-1 Real-Time PCR Kit. 62 HIV-1 RNA negative clinical **serum** specimens and 48 HIV-1 RNA negative clinical **EDTA plasma** specimens were used.

None of the HIV-1 negative clinical specimens gave positive test result for HIV-1 RNA. Diagnostic specificity of RTA HIV-1 Real-Time PCR Kit is 100 %. All of the Internal Controls of tests gave positive result.

Performance Characteristics

Cross Reactivity

To examine the specificity of an assay, cross-reactivity studies should be performed for potential cross-reactive markers. In this study, the specificity of the assay was evaluated by testing 7 reference organism and 9 clinical specimens which were positive. RTA HIV-1 Real-Time PCR Kit do not show any cross-reactivity with other potential cross-reactive markers given in the table below:

Organism	***Source***	**Test Result**
Cytomegalovirus (CMV)	Acrometrix (Cat. No: 94-2014)	Negative
Human Herpes Simplex virus type 1 (HSV-1)	NIBSC (Cat. No: 08/224)	Negative
Human Herpes Simplex virus type 2 (HSV-2)	NIBSC (Cat. No: 08/226)	Negative
Epstein-Barr Virus (EBV)	NIBSC (Cat. No: 08/316)	Negative
Hepatitis B virus (HBV)	NIBSC (Cat. No: 97/750)	Negative
Hepatitis C virus (HCV)	NIBSC (Cat. No: 06/100)	Negative
Mycobacterium tuberculosis	ATCC (25177)	Negative
HTLV-1(3 samples)	Clinical specimens	Negative
Rous sarcoma virus	Clinical specimens	Negative
Parvovirus B19	Clinical specimens	Negative
Epstein-Barr Virus (EBV)	Clinical specimens	Negative
Hepatitis C virus (HCV) (2 samples)	Clinical specimens	Negative
Cytomegalovirus (CMV)	Clinical specimens	Negative

Performance Characteristics

Cross-Contamination

In this study, cross-contamination between samples was evaluated. To do this, five different runs were performed. In every run, 4 high positive HBV sample and 4 HIV-1 negative samples were used. Samples were extracted by RTA Viral Nucleic Acid Isolation Kit according to RTA Viral Nucleic Acid Isolation Kit Handbook. Starting sample volumes were 500 µl and elution volumes were 50 µl. Then, the kit was evaluated accordingly whether or not any cross-contamination was observed.

No cross-contamination was observed during the whole process, and none of the human serum samples exhibited evidence of containing PCR inhibitors as indicated by the amplification of internal control.

Whole System Failure

96 HIV-1 RNA negative clinical **serum** specimens and 36 HIV-1 RNA negative clinical **EDTA plasma** specimens were spiked with WHO International HIV-1 standard (NIBSC code: 97/650) to give a final concentrations of 11 IU/µl in the elution volume which is 3 times the 95% positive cutoff value determined by analytical sensitivity study. Whole system failure rate of RTA HIV-1 Real-Time PCR Kit is ≤1 %.

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RTA.BR.004 Revision Date/Revision No: 09.02.2012/3